OSMOREGULATION IN THE LARVAE AND ADULTS OF THE GRAPSID CRAB *SESARMA RETICULATUM* SAY [1]

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To date, most studies concerned with decapod crustacean larval responses to salinity have concentrated on the determination of optimal salinity-tolerance ranges. While these studies have often demonstrated differential salinity-tolerance ranges for larvae and adults, it is clear that those estuarine species which recruit young by retention within the estuary must possess larval stages that are physiologically adapted for estuarine existence. Although numerous studies have been concerned with osmoregulation in adult crustaceans, especially decapods (for reviews, see Krogh, 1939; Beadle, 1957; Robertson, 1960a; Lockwood, 1962, 1967; Potts and Parry, 1964; Schoffeniels and Gilles, 1970; Prosser, 1973; Vernberg and Silverthorn, 1977) and the need for studies pertaining to all stages of the life cycle has been repeatedly stressed (Prosser, 1957; Vernberg, 1967; Costlow, 1968; Vernberg and Vernberg, 1972), few data exist regarding osmoregulation in decapod larvae. Osmoregulatory adaptations employed by larvae would not, necessarily, reflect those utilized by adults. As planktonic organisms, decapod larvae are at the mercy of currents in their horizontal displacement and may be exposed, therefore, to a completely different set of environmental conditions than benthic, intertidal adult crabs. Surface-area to volume ratios and metabolic rates, both of which may be dependent on size, can interact to dictate the osmotic gradients maintained by larvae. Larvae possess functional antennal glands (Anderson, 1973) but lack gills, important sites of active ion-transport in adult decapods.

Kalber and Costlow (1966) measured the body-fluid concentrations in larvae of *Rhithropanopeus harrisii* for four zoeal and one megalopa stage and examined the effect of eyestalk removal on the osmoregulatory response. Control larvae generally remained hyper-osmotic in salinities from 10 to 30‰ and isosmotic or hyper-osmotic from 30 to 40‰ throughout all of development, except for 24 hours during the second zoeal stage, when they were isosmotic at all salinities. Kalber and Costlow (1968) and Kalber (1970) presented data on osmoregulation in larvae of *Cardisoma guanhumi, Callinectes sapidus, Libinia emarginata* and *Hepatus epheliticus*. No data were presented concerning the actual freezing point depressions of the test media and body fluids, nor were values for means and variation included. However, their graphs of the relationship between internal and external osmotic concentrations indicated the ability of *C. guanhumi* larvae to hyper- and hypo-regulate. The other species were generally hyper-osmotic in sa-

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linities of 10 to 30‰ throughout development. Larvae of *Callinectes sapidus* appeared to hyper-regulate against 40‰ as well, while larvae of *L. emarginata* and *H. epheliticus* seemed to hypo-regulate at this salinity.

Since molting has been shown to affect the osmotic pressure of body fluids in crustaceans (Baumberger and Olmsted, 1928; Baumberger and Dill, 1928; Huf, 1933 as cited by Krogh, 1939; Prosser, Green, and Chow, 1955; Robertson, 1960b; Crowley, 1963; Lockwood and Andrews, 1969; Lindqvist, 1970; Mantel, 1975), the rapid molting cycles in decapod larvae might be reflected in cyclic changes in blood osmotic pressure during each zoeal stage throughout development. In support of this proposition, Kalber and Costlow (1966, 1968) claimed that their data indicated that the blood osmotic pressures in *R. harrisii* and *C. guanhumi* increased as each larval molt was approached and decreased within 12 hours afterward. This tendency was proposed as a mechanism to provide an osmotic gradient necessary to insure water influx at ecdysis, thereby allowing the animal to grow. Proximity to the molt was reflected in increased levels of free amino acids in larval *Callinectes sapidus* (Tucker and Costlow, 1975), but it was unclear whether the observed changes were secondary to possible hemolymph inorganic-ion changes associated with the molt or due to a more direct hormonal effect on amino acid metabolism.

In the interest of following changes in the osmoregulatory capabilities through larval development into adulthood, especially in view of the relative scarcity of data concerning osmoregulation in decapod larvae, the present study was undertaken to trace the ontogeny of osmoregulation in the estuarine, grapsid crab, *Sesarma reticulatum* Say. In addition, special emphasis has been placed on elucidating the possible effects of the molting cycle on larval osmoregulatory capabilities.

The grapsid crab *Sesarma reticulatum* Say is a common species in many salt marshes and estuaries from Massachusetts to Texas (Abele, 1973). It can be found under logs (Herreid, 1969) and burrowing into muddy areas, such as wave-cut marsh faces and higher banks of the marsh (Allen and Curran, 1974), especially where the sand content of the substratum is <10% (Teal, 1958). Throughout its range, the crab is found intertidally (Gray, 1957; Crichton, 1960; Bliss, 1968), often alongside *Uca pugnax* (Teal, 1958; Allen and Curran, 1974) and *Uca minax* (Gray, 1957). Little data on the temperature and salinity ranges experienced by *S. reticulatum* in the field are available. Teal (1959) found the crab active on cloudy days and when the tide was high. Allen and Curran (1974) found specimens in full-strength sea water behind Shackleford Banks, North Carolina, and Pinschmidt (1963), working nearby in the Newport River Estuary, found gravid females in areas where mean monthly salinities ranged from 0.3 to 33.3‰.

Development in *S. reticulatum* was examined by Costlow and Bookhout (1962), who followed the complete larval development and described three zoeal stages and one megalops stage. In another study, Costlow (1966) found that for control animals reared at 25° C in 25‰ sea water, zoeal development was completed in 9 to 11 days. The first stage lasted approximately 3 days and first crab was reached from 18 to 24 days after hatching. Larval temperature and salinity tolerances must be inferred from field studies, as laboratory data in this respect are lacking. Larvae of *Sesarma reticulatum* have been found in the plankton in the Newport River Estuary in North Carolina from May to September at temperatures

and salinities ranging from 19 to 34° C and 18 to 36‰, respectively (Pinschmidt, 1963). Most larvae, however, were found between 25 and 31° C and from 25 to 36‰. Nearly all of the larvae were found in bottom samples and a differential pattern of a higher and narrower salinity range for each larval stage was noted. Tagatz (1968) found *Sesarma* larvae from 3 to 40 km above the mouth of the St. Johns River in Florida. The specimens of *Sesarma reticulatum* and *S. cinereum* were not distinguished and the larvae were grouped as *Sesarma* spp. From 3 to 11 km above the mouth of the river, where salinities ranged from 12 to 36‰, *Sesarma* spp. was second to *Uca* spp. in being most numerous. Forty km above the mouth, the salinity ranged from 9 to 27‰, and *Sesarma* spp. ranked third in abundance behind *Uca* spp. and *Rhithropanopeus harrisii*. Approximately 48 km above the mouth of the river, where salinities ranged from 0 to 11‰, the *Sesarma* spp. zoeae that were collected were dead. Dudley and Judy (1971) found *Sesarma* spp. at inshore stations outside Beaufort Inlet in North Carolina, but could find none at offshore stations. Larvae were found to be consistently more numerous at 8 m than at the surface. Sandifer (1973, 1975), working in the York River Estuary where he recorded salinities ranging from fresh to full-strength sea water, collected *S. reticulatum* larvae from June through September in salinities ranging from 2 to 20‰. Few larvae, however, were collected in waters of salinity <10‰, and most were found in the range 15 to 20‰. The total temperature range of collections was 22.8 to 27.9° C. Stage I larvae comprised approximately 73% of those collected, with stages II and III occurring in equal abundance. As Pinschmidt (1963) and Dudley and Judy (1971) found, most larvae occurred near the bottom. Nearly 75% of stage I larvae were collected in bottom samples, and virtually all stages II and III larvae were taken there. Dean (1975), working in abandoned South Carolina rice fields, found *S. reticulatum* larvae always close to the water-sediment interface.

MATERIALS AND METHODS

All specimens of *Sesarma reticulatum* used in experiments were from a single population living in the banks of a muddy ditch in a part of the salt marsh bordering the Ashley River, approximately 9.6 km from the mouth of Charleston Harbor, South Carolina. The population was submerged at high tide for approximately two hours each tidal cycle. The salinity of the water, determined at various times throughout the summer of 1975, ranged from 10 to 16‰. Although *S. reticulatum* is an intertidal crab, the experimental animals were kept completely submerged to eliminate any effects of desiccation. Larvae and adults were maintained, and all experiments conducted, at 25° C under a 14L:10D light regime. Costlow and Bookhout (1962) determined that development in this crab is unaffected by photoperiod. Experimental salinities were obtained by filtering sea water from North Inlet Estuary and diluting it with distilled water or concentrating it by freezing.

Larval stages

Ovigerous females were brought into the laboratory and maintained individually in glass fingerbowls containing 25‰ filtered sea water at 25° C. Following hatching, larvae were transferred to plastic compartmentalized boxes containing

25° C, 25‰ filtered sea water for the duration of the experiments. Freshly hatched *Artemia* nauplii were added daily as a food source. The water was changed every other day as preliminary observations demonstrated no differences in survival than if the water were changed daily.

Adults

Animals were collected, returned to the laboratory and individually placed in glass fingerbowls containing 25‰ filtered sea water at 25° C. The water was changed daily, and animals were not fed during the experiments. Only males and nonovigerous females in the intermolt condition were used, as disturbances in blood osmotic pressure may be caused by the molt cycle (see introduction for references) and ovigerous animals may have altered blood osmotic pressures (Lindqvist, 1970). Although Gilbert (1959) and Tan and van Engel (1966) demonstrated different osmotic concentrations for males and females in *Carcinus maenus* and *Callinectes sapidus,* respectively, the sexes were not differentiated for data analysis in this study unless evidence appeared during the course of the experiments indicating that it was necessary to separate the results. Specimens ranged in size from 1.9 to 5.1 g, although the majority were approximately 3 g. Since Gilbert (1959) and Lindqvist (1970) have demonstrated that size may have a definite influence on blood osmotic pressure, an attempt was made to use a similar range of sizes in all experiments. Adults used in all experiments conformed to these criteria and were maintained as described.

Acclimation-time tests

When measuring the osmotic concentration of blood, it is necessary to know whether adaptation to a given salinity has been complete (Spaargaren, 1971). Therefore, the rates of salinity adaptation in the larvae and adults were determined before starting the measurements of blood osmotic concentrations.

Larvae. Stages I and III larvae, reared in 25° C, 25‰ filtered sea water, were used. Salinity-tolerance experiments have indicated that complete zoeal development is possible in a range of salinities from 10 to 40‰ (Foskett, 1977). Since both test salinities are equally different from the acclimation salinity and preliminary observations indicated that 10‰ was the most stressful salinity, determination of the time period required for the body fluids to reach osmoregulatory equilibrium following acute exposure was conducted in 10‰ test-medium. It was also assumed that stage II larvae would require an equilibrium period comparable to stages I and III.

Several larvae were placed in 10‰ test-medium and body-fluid samples taken at half-hour intervals until an equilibrium was established. At least three larvae were used at each time. Hemolymph samples were obtained as follows: the zoea was removed from the rearing medium and placed in a small pool of mineral oil, through which it was gently pushed with a pair of micro-forceps to remove some of the water clinging to the body. The zoea was removed from the mineral oil, blotted on filter paper, transferred to a small plastic petri dish and covered with mineral oil. Only larvae which displayed a beating heart and an undamaged cuticle, and to which no water still adhered, were used. Working under a dissecting

scope, mineral oil was sucked up into a finely drawn-out glass capillary. The capillary was then inserted through the membrane between the carapace and first abdominal segment, slid along the underside of the carapace until the heart was reached, and the heart pierced. The hemolymph usually flowed readily into the capillary. This method is quite effective because it always provides a sufficient amount of hemolymph and one can be sure of what is actually drawn by the micropipette. Approximately 50–100 nannoliter volume of hemolymph can easily be obtained. More mineral oil was then sucked up to bracket the hemolymph sample. Freezing-point depressions were determined with a cryoscope modified after that described by Ramsay and Brown (1955) and according to the procedures they outline. Preliminary experiments revealed that, in some instances, leeching of osmotically-active substances by or from the glass capillary took place if the sample was allowed to sit at room temperature for 7 hr. In no case was leeching detected if the freezing point of the sample was determined within 2 hr of sampling. Therefore, all determinations were made within 2 hr of sampling. Usually the freezing point of each sample was measured only once.

Adults. Acclimation-rate determinations for adults were initiated by placing 20 specimens in each of the highest and lowest tolerated salinities, determined to be 50 and 5‰, respectively (Foskett, 1977). Following the procedure described by Barnes (1967), a tolerated salinity is determined as one in which at least half of the specimens had survived to the end of an 8-day period. Blood samples were taken from four animals at the beginning of the experiment and from four different animals in each of the two test salinities after 12 hr exposure. Blood was obtained by inserting the needle of a 1 cc disposable syringe into the arthrodial membrane at the base of a walking leg and withdrawing a sample. The blood was discharged onto a plastic dish and stirred slightly with the needle. In those few instances when a clot would form, only the serum was used. There is good evidence that there is an insignificant osmotic difference between whole blood and serum of crabs (Prosser *et al.,* 1955; Gross, 1964). Fifty μl of blood (or serum) were diluted with 200 ml of distilled water and the osmolality determined on a Fiske osmometer (model OS). The instrument was calibrated against standard NaCl solutions. The average of four determinations for each sample was used in computations to account for variability within the machine itself. The crabs were weighed, discarded, and the procedure repeated with fresh crabs 12 hr later and every 24 hr for a total of four days. In this manner, the amount of time required to establish osmoregulatory equilibrium was determined. It was assumed that the times required for acclimation to the intermediate salinities would be less than the longest time to either of the extremes.

Osmotic regulation in various salinities

Zoeae. Preliminary observations determined the time-course of larval development under the previously described rearing conditions. This information was necessary in order to be able to accurately predict the proper times to sample the body fluids. To trace ontogenetic changes in blood concentrations as well as changes in the blood concentrations associated with each larval molt, hemolymph was extracted and the freezing-point depressions ($\Delta i ° C$) determined at ten dif-

ferent times during the three zoeal and one megalops stages. The sampling times were as follows: immediately after hatching, mid-way through each zoeal stage, and immediately prior to and following each ecdysis. Larvae were considered to be "immediately prior" to ecdysis if at least 50% of the larvae of a hatch had already molted into the next stage. Larvae that were sampled "immediately following" ecdysis were seen to have molted within 15 min prior to exposure to the test salinities. At each of the ten sampling times, from three to six larvae were placed in each of five test salinities of approximately 10, 20, 25, 30, and 40‰, for a period equal to or greater than the equilibrium period determined previously.

Extraction of body fluids and determination of blood freezing-point depressions ($\Delta i° C$) were carried out as already described. The freezing-point depressions of the test salinities ($\Delta e° C$) were also measured throughout the course of the experiment. The entire experiment was conducted twice on separate hatches.

Blood freezing-point depressions ($\Delta i° C$) were plotted against test-media freezing-point depressions ($\Delta e° C$) for each sampling time for each hatch. The least-squares method was used to construct regression lines through the data points, and the lines were tested for degree of linear fit by one-way analysis of variance ($\alpha = 0.05$). To determine if any significant differences existed in the osmotic responses between larvae from the two separate hatches, the regression lines representing the same sampling times for both hatches were compared for equality by one-way analysis of variance ($\alpha = 0.05$). If no difference was observed, the data from both hatches were combined for each sampling time for further statistical analysis. To compare the osmoregulatory response over the entire range of test salinities between pairs of sampling times, the regression lines of the sampling times were tested for equality of the entire lines and for equality of the slopes of the lines by one-way analysis of variance ($\alpha = 0.05$). Since differences observed between two regression lines may be caused by differences only at particular salinities, the osmotic responses at individual test salinities between sampling times were also tested for differences. Because the number of larvae used at each sampling time for each hatch was small, the Mann-Whitney test of equality of means ($\alpha = 0.05$) was used (Mann and Whitney, 1947). This was done for hatch 2 larvae only, since test media salinities varied between sampling times in hatch 1, making it impossible to use this test.

Since classification of decapod larval intermolt stages has not received the same degree of consideration as the classification of adult intermolt stages, Drach's classifications of brachyuran intermolt stages, as modified by Passano (1960), are used in figures and tables to denote the body-fluid sampling times. Larvae sampled immediately following ecdysis are designated stage A; those larvae sampled mid-way through a stage as stage C; and those larvae sampled immediately prior to ecdysis as stage D. When necessary to separate the results of the two hatches, they will be denoted as H1 and H2.

Megalops. Megalops in days 18 and 24 of larval life were subjected to 10, 20, 30, and 40‰ test media. From three to five megalops were placed in each test salinity at 25° C for a period greater than four times that determined for stages I and III larvae. Blood samples were obtained as for the zoeae.

Adults. Animals were maintained in the laboratory for six days prior to the start of the experiment as already described. In this case, animals were fed chopped

fish until three days before the start of the experiment. Osmoregulatory capabil-
ities over a range of salinities were determined by subjecting five specimens to
each of approximately 5, 11, 18, 25, 37, 45, and 52‰ test media at 25° C for a
period equal to the longer of the two acclimation times previously determined. The
blood extractions and osmolality determinations were as described, and animals
were weighed following extractions. The osmolalities of the test media were also
determined. Osmolalities were converted to freezing-point depressions (Δ° C), and
Δi° C, along with the standard errors of the means, plotted against Δe° C.

<div align="center">RESULTS</div>

Zoeae

The time required for the blood of mid-stage I (S1C) and mid-stage III (S3C)
zoeae reared in 25‰ sea water to reach osmoregulatory equilibrium following acute
exposure to 10‰ was 1 hr (Fig. 1). Therefore, before body-fluid sampling, a
minimum of 1 hr was allowed for larvae in all other test salinities.

Larvae of *Sesarma reticulatum* are hyper-osmotic over the salinity range 10
to 35‰ and hyper- or isosmotic at 40‰ throughout zoeal and early megalopa life
(Figs. 2–5). Hyper-regulation is most pronounced at 10‰ and decreases linearly
to near isosmoticity at 40‰. The gradient maintained between the blood and
external medium by individuals at 10‰ varied from 0.16 to 0.71 Δ° C, correspond-
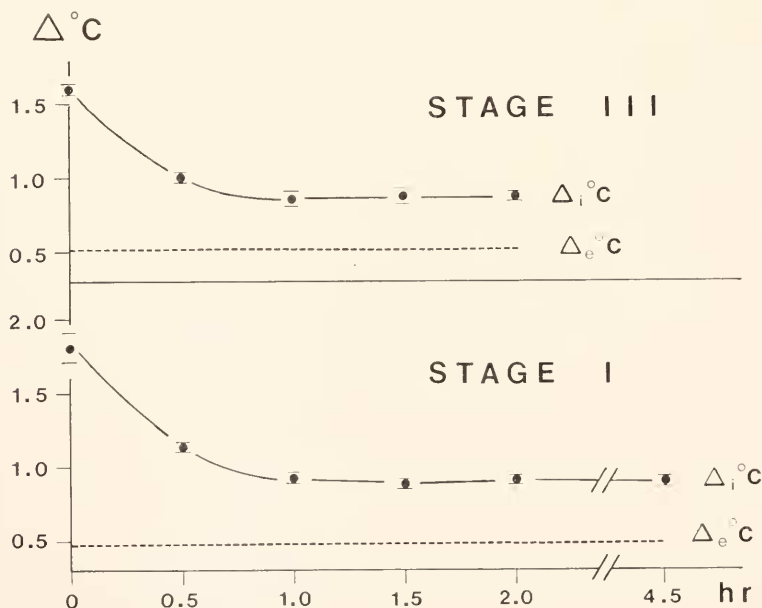

FIGURE 1. Time course of osmotic equilibrium in blood of mid-stage I (S1C) and mid-
stage III (S3C) zoeae following sudden transfer from 25‰ rearing medium to 10‰; each dot
represents the mean of at least three individuals; bars indicate ± s.e.m.

Table I

Regression lines derived from the osmotic responses of larvae sampled at eleven times during development. In those cases where no significant differences ($\alpha = 0.05$) could be detected between hatches, the regression line of the combined data, along with the P-value representing the degree of difference between the two lines, is given. See text for explanation of body-fluid sampling times.

Body-fluid sampling time	Regression lines of blood concentrations over the salinity range 10 to 40‰			
	Hatch 1	Hatch 2	Combined hatches	(*P*-value)
Post-hatch (S1A)	y = 0.73x + 0.63	y = 0.78x + 0.60	y = 0.76x + 0.61	(>0.25)
Mid-stage I (S1C)	y = 0.74x + 0.68	y = 0.79x + 0.62	y = 0.74x + 0.68	(>0.25)
Premolt stage I (S1D)	y = 0.63x + 0.83	y = 0.81x + 0.48		(<0.025)
Post-molt stage II (S2A)	y = 0.87x + 0.40	y = 0.87x + 0.44	y = 0.87x + 0.43	(>0.50)
Mid-stage II (S3C)	y = 0.94x + 0.31	y = 0.72x + 0.69		(<0.0005)
Premolt stage II (S2D)	y = 0.87x + 0.42	y = 0.77x + 0.56	y = 0.82x + 0.50	(>0.25)
Post-molt stage III (S3A)	y = 0.86x + 0.35	y = 0.82x + 0.45	y = 0.84x + 0.40	(>0.10)
Mid-stage III (S3C)	y = 0.89x + 0.36	y = 0.70x + 0.67	y = 0.79x + 0.52	(>0.05)
Premolt stage III (S3D)	y = 0.78x + 0.49	y = 0.79x + 0.55	y = 0.78x + 0.52	(>0.10)
Post-molt megalops (MA)	y = 0.85x + 0.39	y = 0.88x + 0.35	y = 0.86x + 0.37	(>0.75)

ing to 3 to 13‰. Regression lines depicting the relationship between Δi and Δe were tested for linearity and found to be significantly linear at all sampling times for both hatches (*P*-values for the ten sampling times for each hatch ranged from >0.10 to >0.95).

No significant differences (*P*-values ranged from >0.05 to >0.75) existed between lines representing the same body-fluid sampling time for the two hatches for all sampling times except pre-ecdysial stage I (S1D) ($P < 0.025$) and mid-stage II (S2C) larvae ($P < 0.0005$) (Table I). This indicates that the effects of variability due to inherent differences in the two hatches were insignificant except at these two times. For those times when no differences could be detected between the hatches, the data were pooled for further analysis. Therefore, the regression lines in Figures 2–5, excluding those representing the pre-ecdysial stage I and mid-stage II responses, represent the pooled data of the two hatches.

To assess the effects of molting on body-fluid concentrations, differences in the osmotic responses between consecutive sampling times were examined by testing the regression lines and the slopes of the lines for equality. Based on the hypothesis that osmotic pressures increase as ecdysis is approached, the null hypothesis was that the response at the sampling time closer to the next molt was greater than the response at the sampling time not as near the upcoming ecdysis. Therefore, the null hypothesis was that, for each larval stage, the mid-stage response was greater than the post-molt response, and the premolt response was greater than both the mid-stage response and the post-molt response of the next stage. This

was done for 12 pairs of times. Differences in the lines representing the osmotic responses over the entire range of test salinities were detected for only five pairs of times.

Premolt stage I larvae of hatch 2 and mid-stage I larvae exhibited significantly different responses ($P < 0.05$). Contrary to the predicted premolt increase in blood concentration, mid-stage I larvae maintain a greater degree of hyper-osmoticity over the entire range of salinities, although the Mann-Whitney test could detect a difference only at 30‰ for hatch 2 larvae.

Premolt stage I larvae of hatch 1 and post-molt stage II larvae differ significantly ($P < 0.0005$) in their osmotic responses, although no differences ($P > 0.25$) exist in the responses of the post-molt stage II larvae and premolt stage I larvae of hatch 2. This difference is due to a greater degree of hyper-osmoticity by the premolt larvae below $\Delta e° C = 1.65$. In more concentrated media, the post-molt larvae maintain a greater degree of hyper-osmoticity. The Mann-Whitney test indicates that a significant difference between responses exists only at 10‰ ($P = 0.05$), suggesting that the observed difference in the lines is largely due to differences in the responses at 10‰. The slopes of the lines also differ significantly ($P < 0.0005$). The slopes of the regression lines are a measure of the degree of osmotic regulation. As regulation, (*i.e.*, independence of the internal concentration to changes in the medium salinity) increases, the slope of the line will depart from 1.0 and approach 0. Therefore, the premolt larvae exhibit a higher degree of osmotic regulation than do the post-molt larvae.
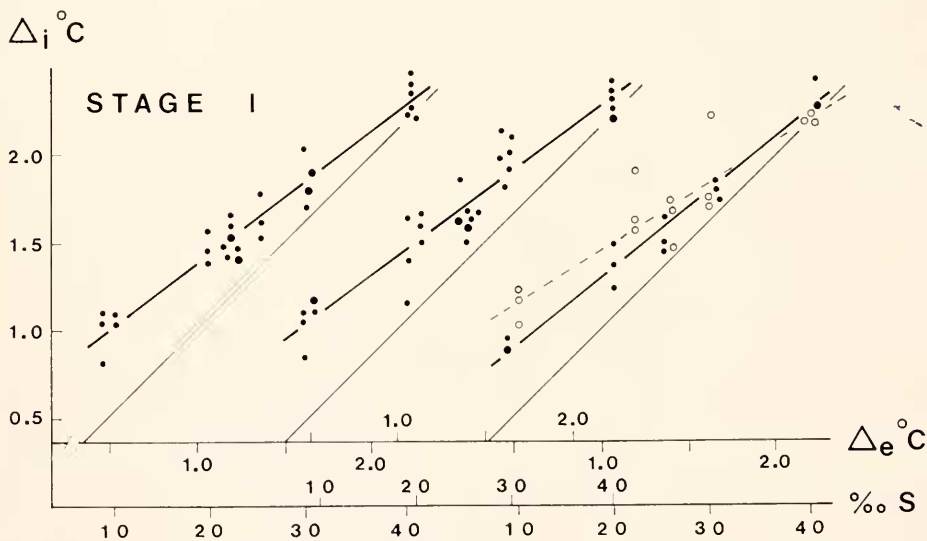

FIGURE 2. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for zoeae sampled immediately following hatching (S1A), mid-way through stage I (S1C) and immediately before first ecdysis (S1D); smaller points represent one individual, larger points represent identical responses by two individuals. Regression lines are derived from combined data of both hatches except for S1D larvae, where open circles represent responses of H1 larvae.
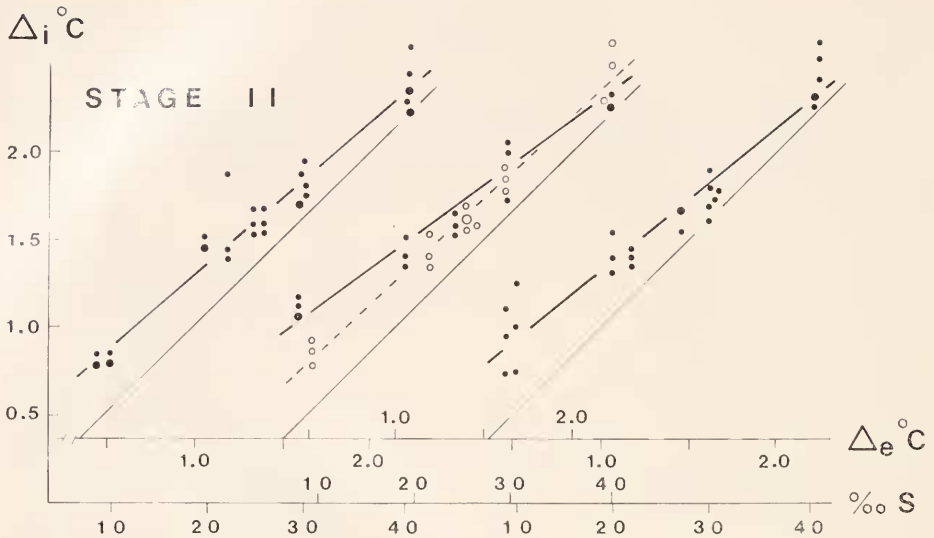
FIGURE 3. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for zoeae sampled immediately following first ecdysis (S2A), mid-way through stage II (S2C) and immediately before second ecdysis (S2D); smaller points represent one individual, larger points represent identical responses by two individuals. Regression lines are derived from combined data of both hatches except for S2C larvae, where circles represent responses of H1 larvae.

Mid-stage II larvae of hatch 2 also differ significantly ($P < 0.005$) from the post-molt stage II larval response. However, no differences could be detected between the responses of post-molt stage II larvae and mid-stage II larvae of hatch 1. Hatch 2 larvae mid-way through stage II display a greater degree of hyper-osmoticity than post-molt stage II larvae up to $\Delta e^\circ\ C = 1.75$. The Mann-Whitney test indicates a significant difference only at 10‰ ($P = 0.05$) suggesting, again, that the observed difference in the lines is largely due to differences in the response at 10‰. The slope of the regression line representing the response of the mid-stage II larvae is significantly less ($P < 0.005$) than that of the post-molt larvae, indicating a greater degree of osmotic regulation by the former.

The response exhibited by premolt stage II larvae is barely significantly different ($P < 0.05$) from that displayed by mid-stage II larvae of hatch 2, although the Mann-Whitney test could detect no differences between the responses of these two groups in hatch 2 at any salinity. The mid-stage II larvae are more hyper-osmotic up to $\Delta e^\circ\ C = 1.98$ and display a smaller slope ($P < 0.05$), indicating a slightly greater degree of osmotic regulation.

Premolt stage II and post-molt megalops larvae differ in their osmotic responses ($P < 0.05$), premolt stage II larvae displaying greater hyper-osmoticity up to $\Delta e^\circ\ C = 1.87$. Mann-Whitney tests performed on hatch 2 larvae indicate that the responses exhibited by premolt stage III larvae are significantly greater ($P = 0.05$) at 10, 20, and 25‰. The slopes of the lines are barely significantly different ($P < 0.05$), indicating that the premolt stage III larvae exhibit a slightly higher degree of osmotic regulation than post-molt megalops.

Megalops

Determination of the osmoregulatory responses of megalops was conducted for megalops in days 18 and 24 of larval life. As shown in Figure 5, megalopa remain hyper-osmotic over the entire range of test salinities, from 10 to 40‰. For both groups, the degree of hyper-regulation at 20 to 30‰ is similar to the response by the three zoeal stages at these salinities. At 10 and 40‰, however, hyper-regulation is much more pronounced in megalops when compared to zoeal stages. By day 24 a relatively high degree of homeosmosis is maintained between 10 and 20‰, in contrast to younger stages.

Adults

Measurements of the changes in blood concentration after transferring *Sesarma reticulatum* adults from 25‰ to 5 and 50‰ revealed that osmotic equilibrium was achieved immediately in 5‰ and within 72 hr in 50‰ (Fig. 6). Therefore, animals were maintained in all test salinities for 3.5 days prior to body fluid sampling.

Adult specimens of *S. reticulatum* exhibit marked capabilities of osmoregulation (Fig. 7). The blood is hyper-osmotic to the medium in the salinity range 5 to 27.5‰ and hypo-osmotic above 27.5‰. The maximum gradient sustained between the blood and external medium is 20.1‰ ($\Delta°$ C = 1.08) at 5‰. Over a range from 5 to 32‰, a high degree of homeosmosis is exhibited. Over this range, while the external medium varies approximately 26.4‰ ($\Delta°$ C = 1.43), the internal concentration varies only 2.5‰ ($\Delta°$ C = 0.14), from 25.6 to 28.1‰. At an external
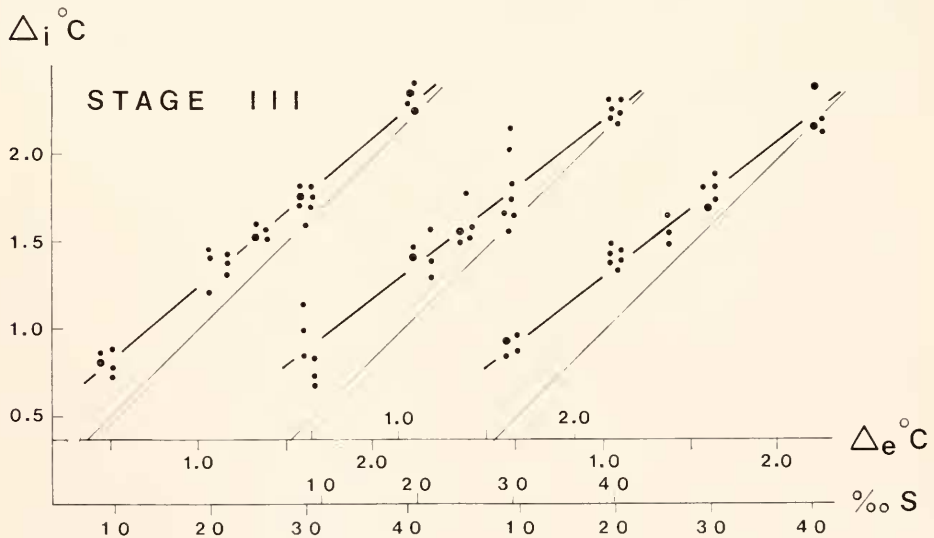

FIGURE 4. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for zoeae sampled immediately following second ecdysis (S3A), mid-way through stage III (S3C) and immediately before third ecdysis (S3D); smaller points represent one individual, larger points represent identical responses by two individuals. Regression lines are derived from combined data of both hatches.
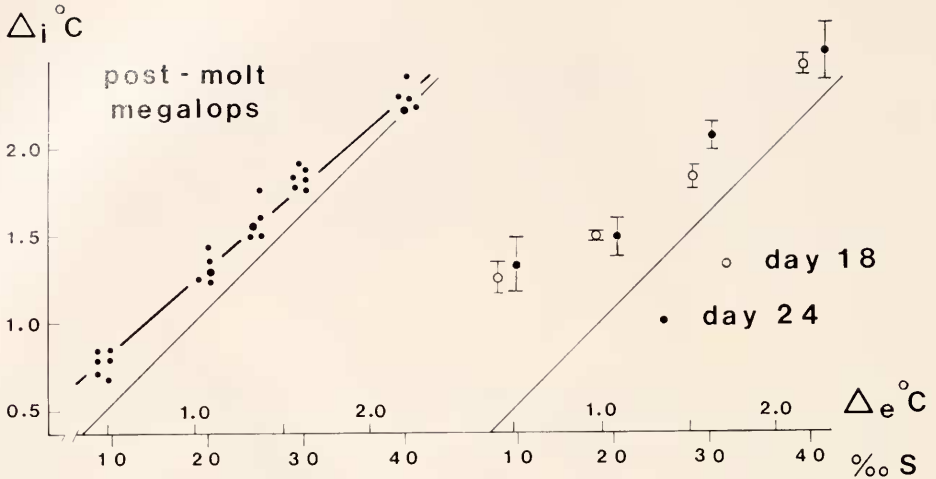
FIGURE 5. Osmotic concentration of the blood as a function of the osmotic concentration of the medium for megalops sampled immediately following third ecdysis (MA) and at days 18 and 24 of larval life. For MA larvae, smaller points represent one individual, larger points represent identical responses by two individuals; regression line is derived from combined data of both hatches. For 18 and 24 day old larvae, each point represents the mean of at least three individuals; bars indicate ± s.e.m.

concentration of $\simeq 32\%_c$, this homeostasis rapidly disappears and the blood is maintained at a nearly constant difference ($\simeq 5.6\%_c$) less than the external medium up to $45\%_c$. Maximum hypo-osmoticity is displayed at $52\%_o$ sea water, where the sustained gradient is $\simeq 7.9\%_c$. No significant differences between males and females or between animals of different sizes were detected.

DISCUSSION

The pattern of osmoregulation in larvae of *S. reticulatum* is apparently established before hatching and varies little throughout larval development. The concentration gradient sustained between blood and medium is maximum at $10\%_o$ and decreases linearly to near isosmoticity at approximately $40\%_o$. No significant degree of homeosmosis is exhibited over any part of the salinity range 10 to $40\%_o$, indicating a relative conformity between salinity variations and changes in body fluid concentrations. While maintenance of a blood concentration hyper-osmotic to dilute media is a typical response of estuarine and coastal species, larvae of *Sesarma reticulatum* are unusual in maintaining blood osmotic pressures greater than the external medium in salinities as high as $40\%_c$ during parts of larval development. Larvae of another estuarine crab, *Rhithropanopeus harrisii*, also display this specialized ability to hyper-regulate against particularly high salinities throughout most of larval life (Kalber and Costlow, 1966). The degree of hyper-regulation maintained at other salinities is also similar to that of *S. reticulatum* larvae. Larvae of *Callinectes sapidus* also hyper-regulate in $40\%_c$ sea water, but only during the first three zoeal stages and day three of megalops. At other times, they are isos-

motic or hypo-osmotic at this salinity. The gradient sustained between blood and medium at 10‰ is less than that for *S. reticulatum*. During the seventh zoeal stage, the ability to osmoregulate is apparently lost, and the larvae become isos-motic at all salinities (Kalber, 1970). Larvae of the land crab, *Cardisoma guan-humi*, are usually hypo-osmotic at 40‰. During the third zoeal stage they become hypo-osmotic in salinities as low as 20‰ as well. The gradient maintained between blood and medium at 10‰ by larvae in the first two zoeal stages is similar to the response by *S. reticulatum* larvae. In later stages, the sustained gradient is con-siderably diminished (Kalber and Costlow, 1968). Osmoregulation in larvae of two polystenohaline crabs, *Hepatus epheliticus* and *Libinia emarginata*, resembles that of typical estuarine species. To the first day of the third zoeal stage, larvae of *H. epheliticus* remain hyper-osmotic in salinities up to approximately 35‰ and hypo-osmotic in 40‰ sea water. As in *S. reticulatum*, the osmotic response varies little throughout development. Larvae of *Libinia emarginata*, on the other hand, begin zoeal life isosmotic to the medium in all salinities, gradually develop hyper-regulation in salinities less than 27 to 30‰ during the middle of larval life, and again become isosmotic in the third day of megalops development (Kalber, 1970).

Kalber and Costlow (1966, 1968) and Kalber (1970) allowed a 1 hr period of adjustment for larvae of *R. harrisii* and 2 hr for larvae of *C. guanhumi, C. sapidus,* and *L. emarginata* following exposure to test salinities before sampling the body fluids. However, no data showing the time-course of osmotic equilibrium were presented to confirm that equilibrium had, indeed, been attained within those time periods. The change in blood concentration after a change in the salinity of the medium is important in the ecology of a species (Gross, 1957; Kinne, 1963, 1967;
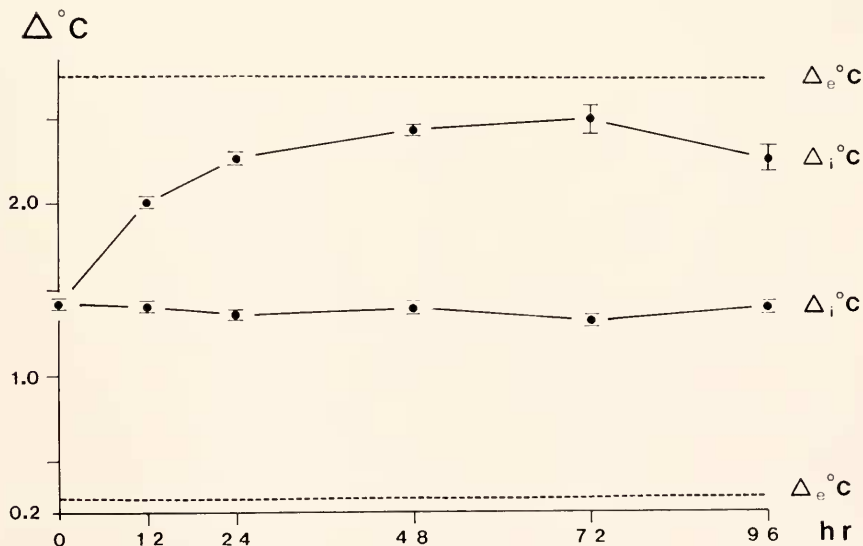

FIGURE 6. Time course of osmotic equilibrium in blood of adult *Sesarma reticulatum* fol-lowing sudden transfer from 25‰ acclimation medium to 5 and 50‰; each dot represents the mean of four individuals; bars indicate ± s.e.m.
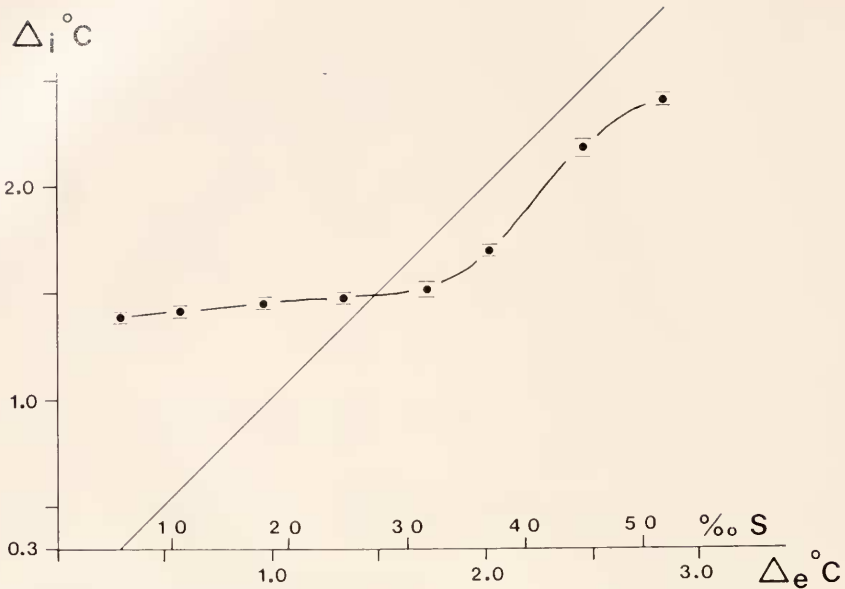
FIGURE 7. Osmotic concentration of the blood of adult *Sesarma reticulatum* as a function of osmotic concentration of the medium; each point represents the mean of five individuals; bars indicate ± s.e.m.

Spaargaren, 1971). Tidal rhythms and sudden heavy rainfalls cause rapid salinity fluctuations to which estuarine organisms are often exposed. Rapid attainment of blood osmotic equilibrium is clearly advantageous to estuarine organisms. However, osmotic adjustments which are too fast will cause blood concentrations to fluctuate back and forth in synchrony to external salinity fluctuations with possible detrimental effects (Kinne, 1967). Results obtained in the present study demonstrate that stages I and II larvae of *S. reticulatum* adjust to salinity changes within 1 hr of exposure. Since the blood concentrations in *S. reticulatum* larvae follow closely changes in the external salinity, the fact that such adjustments are attained rather rapidly suggests that the body fluids are in a constant state of osmotic flux in variable-salinity estuaries. At the tissue level, therefore, not only must the cells exhibit tolerances to wide ranges in concentration, but adjustment of intracellular concentration must be rapid to limit large fluxes of water between blood and cells.

No consistent degree of variation between individuals from the same hatch and same test salinities whose body fluids were sampled at the same time were noted for *S. reticulatum* larvae. Variation in the osmotic responses of individual larvae whose body fluids are sampled at the same time can be caused by differences in size, metabolic rates, or general healthiness. It might also be expected that some degree of variation can exist among individuals as a result of their differing genetic capabilities for osmoregulation (Barnes, 1968). Such differences could exist between individuals hatched from the same female and between individuals hatched from different females. This variation can have important evolutionary and ecological implications in variable-salinity environments (Barnes, 1968). At certain

stages of larval development, more variation was exhibited in moderate than extreme salinities, while the converse was true for larvae sampled at other times. In most instances, no significant differences in the osmotic responses were exhibited by larvae from separate hatches. Only for pre-ecdysial stage I and mid-stage II larvae did significant differences exist between regression lines derived for each hatch. At both of these sampling times, the differences between the hatches were due primarily to differences in the responses at the salinity extremes 10 and 40‰. Differences between the responses of individuals from the two separate hatches at the other sampling times were also due, in large part, to differences in the responses at the extreme salinities. Under short-term, severe salinity-stress, such individual variation could insure that at least some proportion of the larval population would survive, and selection for individual larvae possessing the greatest capabilities for osmoregulation might result in further penetration into the estuary (Barnes, 1968).

In marked contrast to the osmoregulatory responses of the larvae, adult specimens of *S. reticulatum* display impressive powers of hyper- and hypo-regulation over a wide range of salinities. The response is similar to those displayed by some prawns and most crabs of the grapsoid families (see Lockwood, 1962; Panikkar, 1950; and Prosser, 1973, for lists of species). The maximum gradient maintained between blood and medium concentrations by adults in dilute sea water is much greater than for the larval stages and is comparable to that displayed by *Uca crenulata, Pachygrapsus crassipes, Ocypode ceratophthalma* (Jones, 1941; Gross, 1964), and two *Sesarma* species from South Africa (Boltt and Heeg, 1975). At 5‰ there is still no drop in the osmotic pressure to indicate breakdown of the osmoregulatory ability. The degree of hypo-osmoticity remains nearly constant in salinities above 32‰ at a concentration intermediate to that displayed by other hypo-regulators examined (see Gross, 1964; Gross, Lasiewski, Dennis, and Rudy, 1966; and Barnes, 1967).

In light of the differences in the osmoregulatory responses between larval and adult forms, and since adult specimens of *Sesarma* occupy habitats in low-salinity environments in which complete larval development is not possible (see Foskett, 1977), the osmoregulatory capabilities necessary for such penetration into estuaries must unfold during intermediate developmental stages. Although laboratory-reared megalops appear to be more tolerant than zoeal stages (Foskett, 1977), it is apparent that even by late megalops the adult osmoregulatory response is still not attained. Examination of osmoregulation in early juvenile crab stages may reveal osmoregulatory responses that are transitional between the larval and adult forms. This appears to conform to results obtained for other larvae examined. Pearse (1932) found adult specimens of *Cardisoma guanhumi* to be hypo-osmotic in 36‰ sea water, and Quinn and Lane (1966) have demonstrated that *C. guanhumi* is able to regulate Na^+ and K^+ at high levels after seven days exposure to distilled water. Apparently, *C. guanhumi* is a hyper-hypo-regulator. Although Kalber and Costlow (1968) found later stages of *C. guanhumi* larvae to be hypo-osmotic in higher salinities (30 and 40‰), the fact that they were also hypo-osmotic at 20‰ and isosmotic at 10‰ indicates that the adult response is still not attained by the end of zoeal development. Larvae of *Rhithropanopeus harrisii* in the final zoeal stage osmoregulate similarly to earlier larval stages (Kalber and Costlow, 1966), but unlike adults (Smith, 1967). *Hepatus epheliticus,* an osmoconformer

as an adult, remains hyper-osmotic throughout zoeal life (Kalber, 1970). Presumably, larvae nearing megalops and settling crab stages gradually lose this ability, but no data were presented to verify this claim. Larvae of *Libinia emarginata* attain the adult response by gradually becoming isosmotic with the external medium throughout early megalopa (Kalber, 1970). However, since first stage zoeae are also generally isosmotic with external salinities, it cannot be discerned if isosmoticity in later stages simply represents the osmoregulatory pattern of larvae for that stage of development, independent of the future adult response, or if such isosmoticity truly represents transition to adult patterns of osmoregulation. Likewise, megalops of *Callinectes sapidus* osmoregulate similarly to the adults (Ballard and Abbott, 1969; Kalber, 1970) but, again, early zoeal stages also display a similar response. To summarize, then, there appears to be no clear trend toward development of adult osmoregulatory patterns toward the end of larval life. Although later stages of *Callinectes* and *Libinia* display a response similar to adults, even the early stages exhibit responses much like the adults. In forms such as *Sesarma* and *Cardisoma,* where adults display hypo- as well as hyper-regulation and which exhibit significant degrees of homeostasis, the adult osmoregulation pattern is still not established by late megalops. It is interesting, however, that *Cardisoma* larvae possess the ability to hypo-regulate while *Sesarma* larvae do not.

For most species examined thus far, there appears to be a general larval trend of hyper-osmoticity in salinities encountered in nature for most or all of larval life. The significance of this observation is not readily obvious. Kalber and Costlow (1966) and Kalber (1970) propose that hyper-osmoticity in all salinities is a mechanism to provide an osmotic gradient necessary to insure water influx at ecdysis. Kalber and Costlow (1966) sampled daily the body fluids of *R. harrisii* larvae throughout zoeal development and claimed to detect increased hyper-regulation immediately before and up to 12 hr after each molt. Since a minimum of data was chosen to be presented and differences between regression lines and responses at individual salinities not statistically analyzed, it is difficult to see clearly that such a pre-ecdysial rise in blood osmotic pressure is a general larval trend. Examination of the available data indicates that hyper-regulation in *R. harrisii* was more pronounced immediately prior to ecdysis only at 40‰, a salinity which is probably never encountered by larvae of this species. Data for other species (Kalber and Costlow, 1968; Kalber, 1970) provide no support for the hypothesis that increased hyper-osmoticity is necessary for water influx at the molt. To actually test the validity of such a hypothesis, the osmotic responses of *S. reticulatum* larvae were determined immediately before and after ecdysis and mid-way through each stage. If Kalber's hypothesis is correct for *Sesarma* larvae, the body-fluid concentrations immediately before ecdysis should be greater than those immediately after ecdysis and mid-way through the stage preceding the molt. The results obtained reveal no consistent tendencies of larvae to increase hyper-regulation over the entire range of test salinities immediately before ecdysis compared to other times.

The normal physiology of crustaceans is continually dominated by the molt cycle. Internal changes occurring in the integumental tissues, hepatopancreas, urine and blood (Bursey and Lange, 1971), as well as metabolism, behavior, reproduction and sensory acuity are all affected by the periodic replacement of the

integument (Passano, 1960). Since the molting cycle is so short for crustacean larvae, the changes associated with such growth are magnified in their rapidity. In adults, the various phases of the molting cycle are often reflected in changes in blood composition. As a result, various physiological problems may arise due to variations in ionic ratios and total ionic concentrations, and from dilution and changes in body surface permeability (Lockwood, 1967). Baumberger and Olmsted (1928), working with *Pachygrapsus*, were the first to notice differences in the blood osmotic pressures between different stages of the molt cycle. They noted an increased blood osmotic pressure immediately before ecdysis and a subsequent drop following the molt. They postulated that such a rise before ecdysis was responsible for water uptake necessary for growth. However, Robertson (1960b) has criticized the techniques utilized and the significance of their findings. In another study, Baumberger and Dill (1928) found an increase in blood osmotic pressure during the act of molting in *Callinectes* and, again, postulated that such a rise could account for water uptake at the molt. Since then, others have noted similar pre-ecdysial rises in blood osmotic pressure (Parry, 1953; Robertson, 1960b; Lockwood and Andrews, 1969) but have demonstrated that such rises are insufficient to account for the water uptake at the molt. There are exceptions to the general rule of premolt increases and subsequent post-molt decreases in blood osmotic pressures. Crowley (1963) found an average 12 per cent decrease in total cation concentration in the premolt period as compared to the normal late intermolt levels in several species of crayfish. Lindqvist (1970) found two species of terrestrial isopods which displayed lower blood osmotic pressures during molting than did nonmolting animals. Although Parry (1953) found a premolt rise in blood osmotic concentrations in *Ligia oceanica*, there was no indication of a sudden uptake of water after the molt. Post-molt animals had higher blood osmotic concentrations than premolt animals and even animals sampled four days after the molt showed no subsequent drop in osmotic pressure. Since cells are generally assumed to be isosmotic with the blood (Schoffeniels and Gilles, 1970) and organic compounds account for about half of the total intracellular osmotic pressure in crustaceans, changes in hemolymph concentrations associated with the molt should be reflected in changes in the levels of these organic constituents, especially the free amino acids. Contrary to such expectations, Dall (1975) found no significant changes in the levels of leg muscle and blood ninhydrin-positive substances during the molt cycle in the rock lobster, *Panulirus longipes*. In *Gecarcinus lateralis*, the total free amino acid content in the claw muscle decreased as the molt was approached and increased during the post-molt period (Yamaoka and Skinner, 1976). The decreased premolt concentrations could not be explained by dilution of the body fluids and tissue hydration.

Clearly, the changes in the blood resulting from the molting cycle vary between species and may be complicated by such factors as water absorption during and after the molt, the amount of stored substances in the tissues, and the relative requirements for these stores in providing energy and material for the new exoskeleton (Florkin, 1960). With respect to *S. reticulatum* larvae, an osmotic gradient between blood and medium may well be necessary to insure water influx at the molt. However, there would seem to be little need to raise the pre-ecdysial blood concentration, since the blood is already hyper-osmotic to the entire range of salin-

ities in which the larvae can survive. The adults of many species display increased concentrations of calcium, proteins, and other ions prior to molting. Most often, the increases are attributed to resorption of these constituents from the old exoskeleton. Therefore, in large part, it is the heavily calcified nature of the exoskeleton of adults which causes large-scale changes in the blood at the molt. The various constituents are resorbed, stored in, and transported by the blood, necessitated by the fact that large amounts of these substances must be readily available for deposition in the new exoskeleton. For larval stages, which lack a heavily calcified exoskeleton, there is no need to resorb various constituents from the old exoskeleton, because sea water probably contains sufficient amounts for deposition in the new one. Larvae would not be expected, therefore, to display increased concentrations of these constituents prior to molting.

Robertson (1960b) has shown that the water taken up at the molt is isosmotic to the medium. Since larvae of *Sesarma* are hyper-osmotic, the rapid uptake of sea water may be expected to dilute the blood during and after the molt. As previously discussed, changes in larval blood concentrations follow changes in the medium concentrations so closely and so quickly (within 1 hr), that a rapid turnover of salts and water between larva and medium is indicated. The mechanisms responsible for maintenance of hyper-osmoticity over the entire range of test salinities are designed to operate successfully despite such a rapid turnover. That no consistent post-molt dilutions were detected for *Sesarma* larvae may be due to the fact that the salt-uptake mechanisms are necessarily adapted to cope with rapid turnovers of water and salts, such as occurs during the molting process. Such osmoregulatory mechanisms are probably under hormonal control. A quantity of evidence supporting the existence of neuroendocrine control of salt and water regulation in adult crabs has emerged (for a review see Kamemoto, 1976). With respect to larval stages, eyestalk removal promotes greater size increases at the molt for *Callinectes* larvae (Costlow, 1963); and Kalber and Costlow (1966) found that larvae of *Rhithropanopeus* with eyestalks removed 12 hr after the first molt lose the ability to hyper-regulate in salinities below 30‰, while increasing hyper-regulation at 40‰. A day later, however, the eyestalkless larvae become hyper-osmotic in salinities ranging from 10 to 40‰ but again lose the ability to hyper-regulate in salinities below 30‰ following the second molt. Therefore, while the trend of decapod larvae to hyper-regulate over the range of salinities encountered in nature may be related to a necessity to maintain an osmotic gradient in order to insure water influx at ecdysis, there is no evidence that increased hyper-osmotic gradients are necessary at the time of molt. Instead, it would appear that neuroendocrine controls are probably more important in insuring water uptake at the molt, possibly through regulations of the extent of body-surface permeability to salts and water, as demonstrated for *Gecarcinus lateralis* (Mantel, 1968).

Since many estuaries are characterized by a two-layered, stratified circulation in which deeper, landward-flowing salt water is overlain by seaward-flowing fresh water the significance of the general larval trend of hyper-osmoticity in salinities normally encountered in nature may be understood if hyper-osmoticity is considered as a mechanism to increase the density of the larva, enabling it to remain close to the bottom, thereby helping to insure retention within the estuary. Since larvae lack a heavy exoskeleton, hyper-osmoticity in all salinities may also be neces-

sary to provide turgor pressure to insure integrity of the thin larval cuticle. This would explain why larvae, including older megalops, display a different osmoregulatory response from adults and suggests that the adult response will be attained only with the appearance of a rigid exoskeleton during the early juvenile crab stages.

Summary

1. Blood osmotic concentrations in the three zoeal stages, megalops stage, and adults were determined over a wide range of salinities for the estuarine, grapsid crab, *Sesarma reticulatum* Say.

2. Larvae are hyper-osmotic over the salinity range 10 to 35‰ and hyper- or isosmotic at 40‰ throughout zoeal and early megalopa life. Older megalops display increased hyper-osmoticity at 10 and 40‰, compared to earlier zoeal stages.

3. Adult specimens of *S. reticulatum* are hyper-osmotic in the salinity range 5 to 27.5‰, and hypo-osmotic in salinities >27.5‰. The adult response is apparently attained during early juvenile crab stages.

4. The effects of molting on blood osmotic concentrations is discussed. The rapid molting cycles of the larvae do not affect the blood osmotic concentrations.

5. The general larval trend of hyper-osmoticity over wide ranges of external salinities may serve to increase the density of the larva, helping to promote retention within the estuary. Hyper-osmoticity may also act to provide turgor pressure to insure integrity of the thin larval cuticle.

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